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Histopathological evaluation of the effect of platelet-rich fibrin on canine cutaneous incisional wound healing

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Abstract

Platelets are a natural source of growth factors and cytokines that promote wound healing, and platelet-rich fibrin contains concentrated growth factors. Growth factors in platelet-rich fibrin may promote wound repair. In this study the effect of platelet-rich fibrin was evaluated in a dog model of cutaneous incisional wounds using histology and semi-quantitative evaluation. One pair of ten-centimeter full-thickness parallel linear cutaneous wounds were created on the backs of 15 dogs on the both sides of vertebral column. On the left side the platelet-rich fibrin clot was applied to the edges of the wound (Treatment group) and the right side received nothing (Control group). All wounds were then closed with 3-0 non-absorbable Nylon suture. The dogs were divided into three groups of five dogs. The wound tissues were sampled by electrosurgery in group one after 3 days, group two after 7 days and group three after 14 days post surgery. For each specimen, histopathological examination and semi-quantitative evaluation was performed by light microscopy using Hematoxylin & Eosin and Masson’s trichrome staining. The results demonstrated that platelet-rich fibrin improved and accelerated cutaneous incisional wound healing.

Keywords: Platelet-rich fibrin, wound healing, cutaneous wound

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Introduction

The use of blood-derived products to seal wounds and stimulate healing began with the use of fibrin glues, which were first described 40 years ago and are constituted of concentrated fibrinogen (polymerization induced by thrombin and calcium). Nowadays, fibrin glues prepared from human plasma, such as Tisseel (Baxte, USA), are widely used. Autologous fibrin glues are considered the best choice to avoid contamination risk, but their use remains very limited owing to the complexity and the cost of their production protocols. Consequently, the use of platelet concentrates to improve healing and to replace fibrin glues, as first described by Whitman *et al.,* has been explored considerably during the last decade, Choukroun's PRF (platelet-rich fibrin) is the latest development of these protocols (Dohan *et al.,* 2008). Platelets are a natural source of growth factors and cytokines that promote wound healing, and platelet-rich fibrin (PRF) contains concentrated growth factors such as platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) (Kimura *et al.,* 2005). Platelet-rich fibrin (PRF) belongs to a new generation of platelet *concentrates*, with simplified processing and without biochemical blood handling (Dohan *et al.,* 2006). The use of fibrin glue or platelet concentrate (often named platelet- rich plasma (PRP)) during surgical procedures is a current treatment concept used to accelerate wound healing and tissue maturation. Choukroun's platelet-rich fibrin (PRF), a second generation platelet concentrate, was defined as an autologous leukocyte and platelet-rich fibrin biomaterial in France by Choukroun *et al.* in 2001. Unlike other platelet concentrates, this technique does not require any anticoagulants or bovine thrombin or any other gelling agent. This open protocol is very simple and inexpensive. Briefly, blood is collected in dry glass tubes or glass-coated plastic tubes and immediately softly centrifuged. Three layers are formed: a red blood cell (RBC) base at the bottom, acellular plasma (platelet-poor plasma (PPP)) as a supernatant, and a PRF clot in the middle (Fig. 1). This clot combines many healing and immunity promoters present in the initial blood harvest. It can be used directly as a clot or after compression as a strong membrane. Potential clinical indications of PRF for humans in oral and maxillofacial surgery are numerous, including, for example, the improvement of soft tissue healing and bone graft protection and remodelling. It is also useful for Schneiderian membrane protection or as a sole osteoconductive filling material during a sinus-lift procedure. In plastic surgery, PRF clots are often directly used to fill cavities or mixed with an adipocyte graft during a lipostructure. Membranes could also be useful for small otologic surgery (Dohan *et al.,* 2010).

Materials and methods

15 healthy 1 year old (13 male and 2 female) mixed breed dogs with 20 kilograms of weight were used in this study. The 15 dogs were randomly divided into three groups of 5 dogs (group A,B,C) and used for wound healing evaluation. Thirty minutes before surgery, the dogs were sedated using a combination of ketamin (5 mg/kg, IM [Ketamin® 10%, alfasan, woerden –Holland]) and acepromazine (0.05 mg kg^{-1}, IM [Neurotran® 1%, alfasan, woerden- Holland]). Anesthesia was induced using thiopental sodium 5% (12 mg/kg, IV [Pentothal®]) and maintained with halothane 1% in oxygen (Fossum *et al.,* 2002). Hairs of the back were shaved and then the region was sterilized with 10% Povidone-iodine and 70% alcohol (Hichman *et al.,* 1995; Fossum *et al.,* 2002). Two 10-cm long parallel full-thickness skin incisions were performed under aseptic conditions on the both sides of vertebral column (Hichman *et al.,* 1995; Fossum *et al.,* 2002; Kimura *et al.,* 2005; Gal *et al.,* 2006). On the left side PRF clot was applied to the edges of the wound (Treatment group) and the

right side received nothing (Control group). All wounds were then closed with 3-0 non-absorbable Nylon suture [Ethilon™] (Fig. 2). The wound tissues were sampled by electrosurgery in group one after 3 days, group two after 7 days and group three after 14 days post surgery (Hichman *et al.,* 1995; Fossum *et al.,* 2002; Kimura *et al.,* 2005). The experimental protocol and animal care were in compliance with the requirements of the Ethics Committee.

Preparation of PRF (4):

For each dog, the blood sample (1 tube of 20 ml each) was obtained from a jugular vein. The blood samples were taken without anticoagulant in dry glass tubes and were immediately centrifuged at 3,000 rpm for 10 minutes with a specific table centrifuge at room temperature (Fig. 1). After centrifugation, the PRF clot was removed from the tube using sterile tweezers, separated from the RBC base using sterile scissors, and placed in a sterile cup (Dohan et *al.,* 2010).

Histological Examination:

The specimens were collected, fixed in 10% buffered formalin and processed for paraffin embedding, sectioned in 5 µm thin slices and stained with Hematoxylin & Eosin (H&E) and Masson's trichrome and examined by pathologist (Bancroft *et al.,* 1982). We were interested in the following histological structures and changes in: the epidermis (re-epithelization and keratinization), the dermis, and the striated muscle (creation of fibrin network, presence of polymorphonuclear leukocytes – PMNL, tissue macrophages, migration, proliferation and orientation of fibroblasts, creation of new extracellular matrix (ECM) – especially new collagen fibres and neoangiogenesis. The histological structures and processes (epithelization, polymorphonuclear leucocyte, tissue macrophages, fibroblasts, new collagen and neoangiogenesis) were semi-quantitatively evaluated in coded slides according to the following scale: 0, 1, 2, 3 (Table 1) (Gal *et al.,* 2006). *Mann-Whitney U* non-parametric tests were used for statistical analysis of differences, with (p < 0.05) considered significant (Kimura *et al.,* 2005). The data obtained from semi-quantitative evaluation of histological changes are presented as mean ± standard deviation (SD). The semi-quantitative evaluation of 3, 7 and 14 days after surgery were compared using *Vidinsky* scales and analyzed by *Mann Whitney U* Test in SPSS version 16 (Gal *et al.,* 2006).

Results

Histopathological examination (Fig. 3) and semi-quantitative evaluation (Table 2 and 3) demonstrated differences in wound healing progress among the three groups.

At day 3, epithelialization was seen on the upper portion of the inner wall of the wound edges in treatment group (Fig. 3 A), while no epithelialization was seen in control group and wound edges were covered by blood coagulum and inflammatory cells (Fig. 3 B); newly organized collagen bundles were not obvious in any group. Deeper portion of the wound was filled with test material in PRF group (Fig. 3 C), while this region had been occupied with loose connective tissue in control group (Fig. 3 D). There was no significant difference between the two groups ($p > 0.05$).

By day 7, there were increased newly organized collagen bundles and relatively advanced epithelium at the wound junction of PRF-treated wounds, demonstrated in Fig. 3 E&G, as compared to non-treated wounds (Fig. 3 F&H). Extension of epithelial tissue, down side the inner wall of the wound edges was prominent in both experimental groups. The wound surface was completely covered by newly formed epithelial tissue in PRF group (Fig. 3 E) while re-epithelialization on wound surface had not been completed in control group, as the epithelia of wound edges were not connected with each other (Fig. 3 F). There was no significant difference between the two groups ($p > 0.05$).

By day 14, Healing sites of the wounds were almost level and there were relatively

advanced epithelium at the wound junction of the wounds in both experimental groups as it was thicker enough in PRF-treated group. The wounds junction were filled with dense collagen bundles as the inner walls of the wound edges were united at all (Fig. 3 J&L). Deeper portion of the wounds contained abundant collagen bundles, though in comparison with untreated wounds, collagen fibers were more abundant in PRF-treated group (Fig. 3 I&K). Rich neovascularisation was found only in the PRF-treated wounds (Fig. 3 I&K). There was no significant difference between the two groups ($p > 0.05$).

Table 1. Explanation of used scale in the semi-quantitative evaluation of histological section*

No.	Epithelization	PMNL	Tissuemacrophages	Fibroblasts	Neo-angiogenesis	Newcollagen
0	Thickness of cut edges	Minimum	Absent	Absent	Absent	Absent
1	Migration of epithelial cells	Mild	Mild	Mild	Mild	Mild
2	Bridging of the incision	Moderate	Moderate	Moderate	Moderate	Moderate
3	Complete regeneration	Marked	Marked	Marked	Marked	Marked

*** The histological structures and processes (epithelisation, polymorphonuclear leucocyte (PMNL), tissue macrophages, fibroblasts, new collagen and neoangiogenesis) were semi-quantitatively evaluated in coded slides according to the following scale: 0, 1, 2, 3.**

Table 2. Semi-quantitative evaluation of histological changes / structures during skin wound healing* (PRF-treated group)

Day	Epithelization	PMNL	Tissue macrophages	Fibroblasts	Neo-angiogenesis	New collagen
3	0.71 ± 0.48	1.57 ± 0.53	0.71 ± 0.48	0.28±0.48	0 ± 0	0 ± 0
7	1.4 ± 0.54	1.6 ± 0.89	1.4 ± 0.54	1.6 ± 0.54	1.4 ± 0.54	1.8 ± 0.44
14	2.8 ± 0.44	1 ± 1	1.2 ± 0.83	1.6 ± 0.54	3 ± 0	2.2 ± 0.44

*** *Mann—Whitney U* non-parametric tests were used for statistical analysis of differences, with (P < 0.05) considered significant. The data obtained from semi-quantitative evaluation of histological changes are presented as mean ± standard deviation (SD). The semi-quantitative evaluation 3, 7 and 14 days after surgery were compared using Vidinsky scales and analyzed by *Mann Whitney U* Test in SPSS version 16.**

Table 3. Semi-quantitative evaluation of histological changes / structures during skin wound healing* (control group)

Day	Epithelization	PMNL	Tissue macrophages	Fibroblasts	Neo-Angiogenesis	New collagen
3	0.33 ± 0.57	1.66 ± 0.57	0.33 ± 0.57	0.33 ± 0.57	0 ± 0	0 ± 0
7	2 ± 0.0	1.8 ± 0.44	1.6 ± 0.54	1.6 ± 0.54	1.2 ± 0.44	1.4 ± 0.54
14	2.33 ± 0.51	0.5 ± 0.83	0.66 ± 0.81	1.5 ± 0.54	2.5 ± 0.83	2.5 ± 0.54

*** *Mann—Whitney U* non-parametric tests were used for statistical analysis of differences, with (P < 0.05) considered significant. The data obtained from semi-quantitative evaluation of histological changes are presented as mean ± standard deviation (SD). The semi-quantitative evaluation 3, 7 and 14 days after surgery were compared using *Vidinsky* scales and analyzed by *Mann Whitney U* Test in SPSS version 16.**

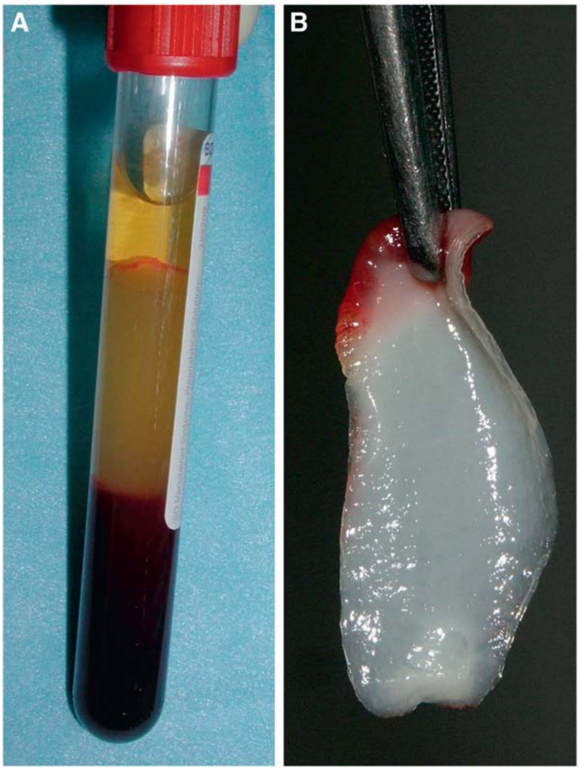


Figure 1. A) After centrifugation three layers were formed: a red blood cell (RBC) base at the bottom, acellular plasma (platelet-poor plasma (PPP)) as a supernatant, and a PRF clot in the middle. B) The PRF clots were collected and changed into membranes.



Figure 2. Ten-centimeter full-thickness parallel linear cutaneous wound on the left and right side of vertebral column(R: Right side, L: Left side). All wounds were closed with 3—0 non-absorbable Nylon suture.

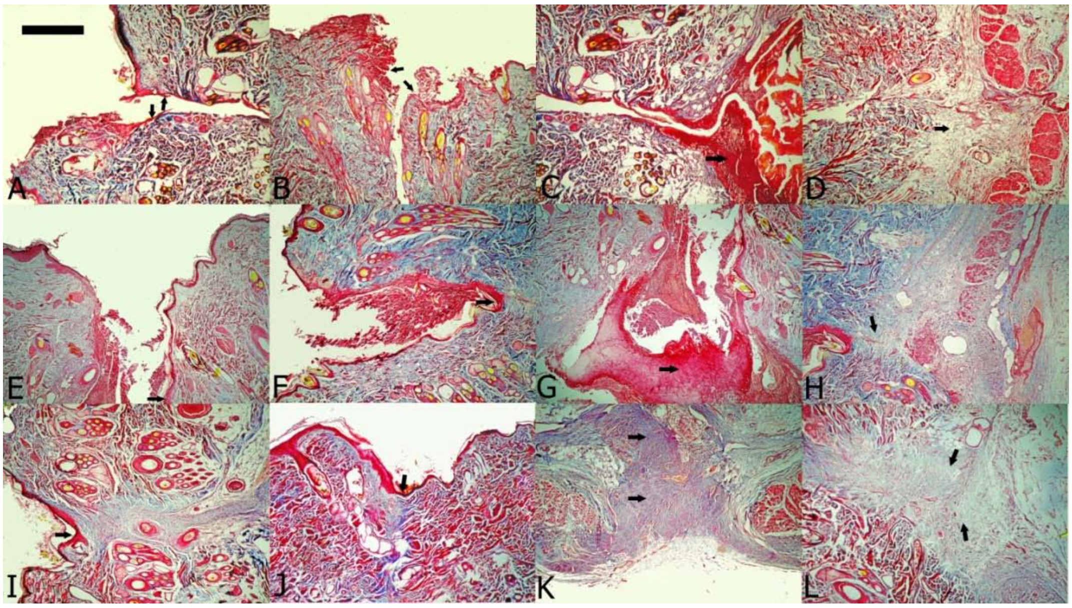


Figure 3. Masson's trichrome stain of wound tissue at day 3 (A-D), 7 (E-H), 14 (I-L) after wounding. (A, B, E, F, I, J) show the superficial portion of the wound. (C, D, G, H, K, L) show the deep portion of the wound. (A&C) Represent sections from the treated wounds at day 3. Epithelialization was seen on the upper portion of the inner wall of the wound edges in PRF-treated group. (B&D) Represent tissues from the untreated wounds at day 3. No epithelialization was seen in control group and wound edges were covered by blood coagulum and inflammatory cells. (E&G) Represent tissues from the treated wounds at day 7. The PRF-treated wound at day 7 has abundant newly organized collagen bundles at the wound junction. (F&H) Represent tissues from the untreated wounds at day 7. New collagen fiber bundles are Sparse. (I&K) Specimens from the treated wounds at day 14. New collagen bundles filled densely. (J&L) Specimens from the untreated wounds at day 14. The wound junction is filled with dense collagen bundles. In all pictures the arrows show the healing site, 40×.

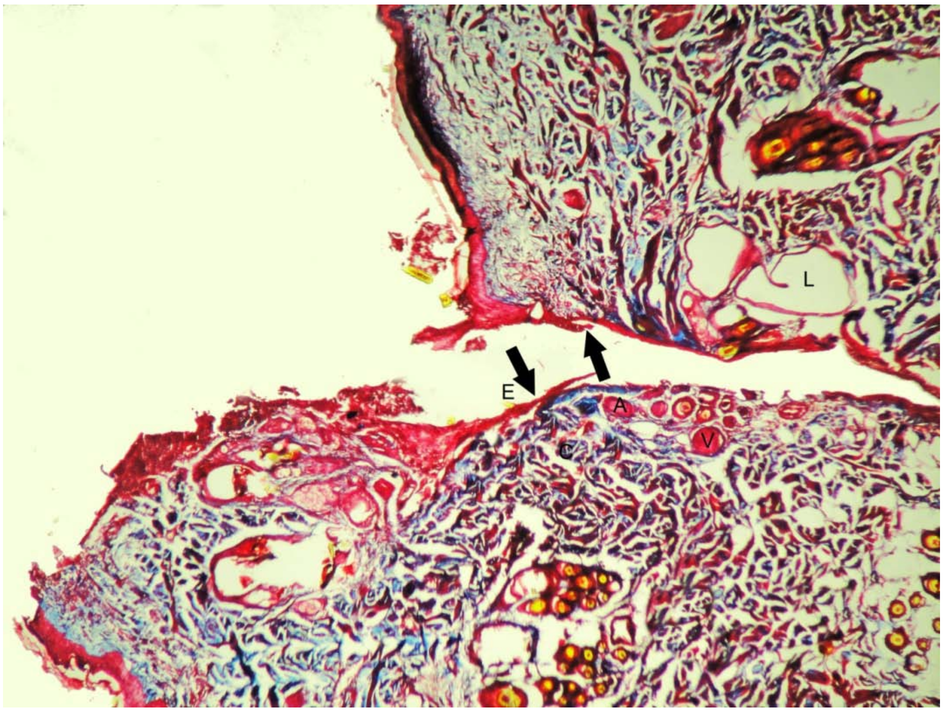


Figure 3.A. Masson's trichrome stain of wound tissue at day 3 after wounding. Representatives sections from the treated wounds. Epithelialization was seen on the upper portion of the inner wall of the wound edges in PRF-treated group. The arrows show the healing site. (E: Epithelium, A: Artery, V: Vein , L: Lymph Node.), 40×.

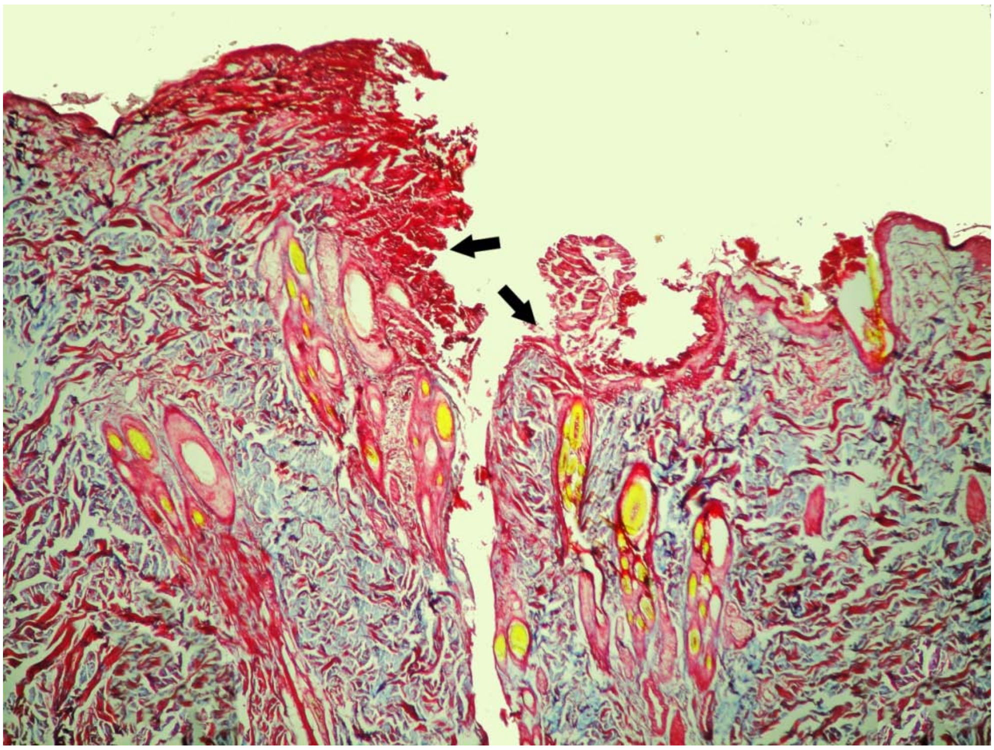


Figure 3.B. Masson's trichrome stain of wound tissue at day 3 after wounding. Representatives tissues from the untreated wounds. No epithelialization was seen in control group and wound edges were covered by blood coagulum and inflammatory cells. The arrows show the healing site, 40×.

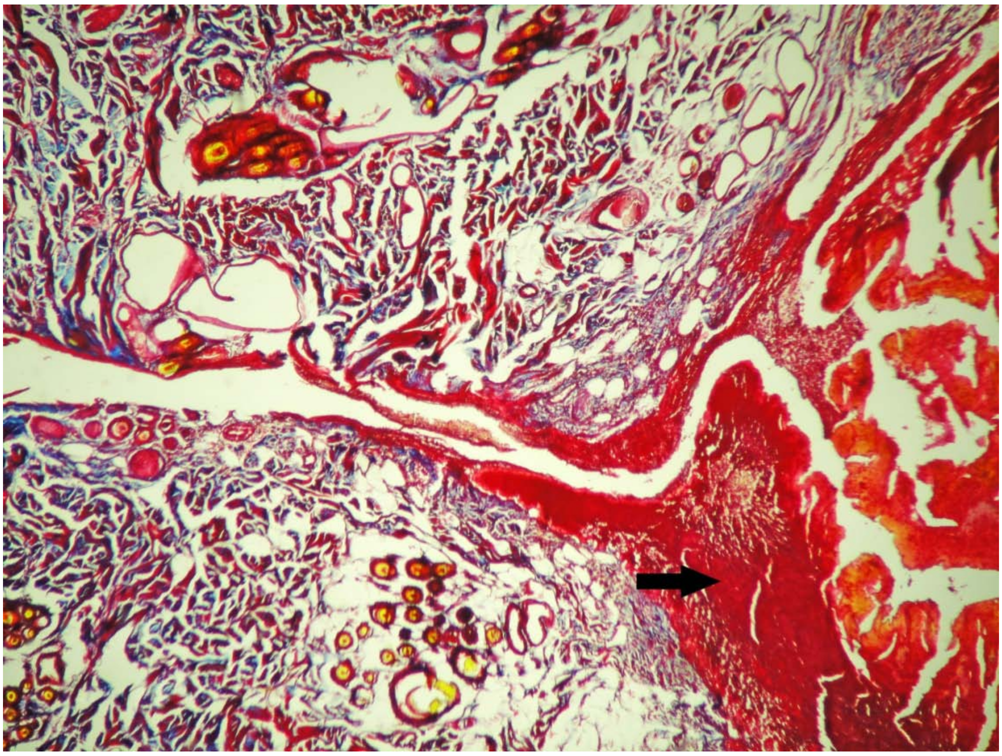


Figure 3.C. Masson's trichrome stain of wound tissue at day 3 after wounding. Representative sections from the treated wounds. The arrow shows the healing site, 40×.

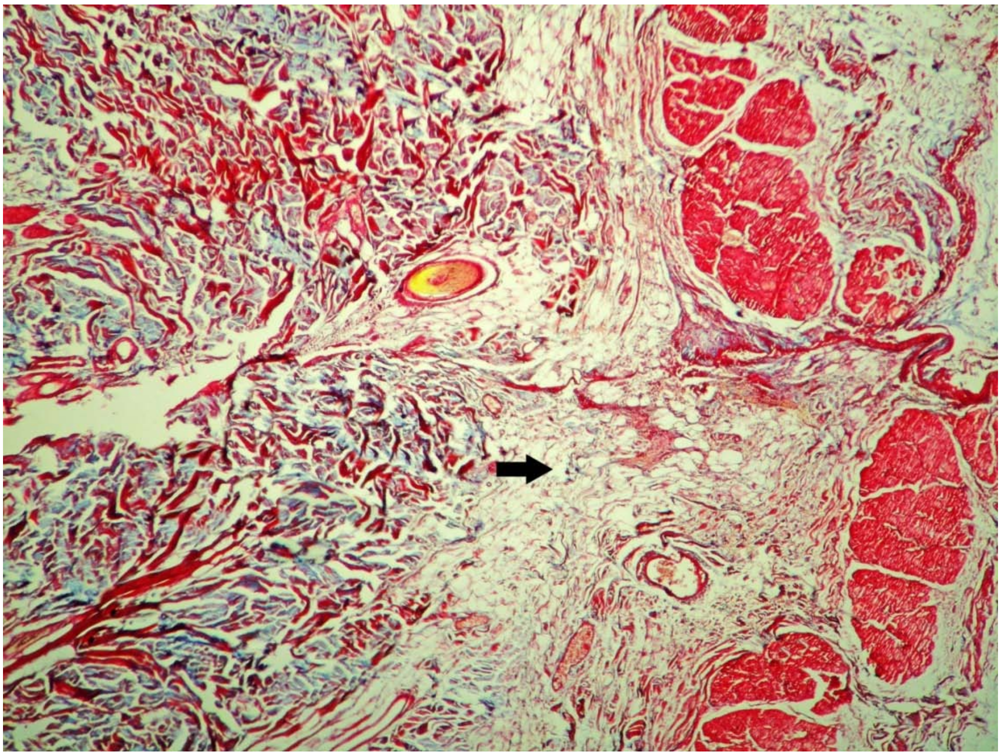


Figure 3.D. Masson's trichrome stain of wound tissue at day 3 after wounding. Representatives tissues from the untreated wounds. The arrow shows the healing site, 40×.

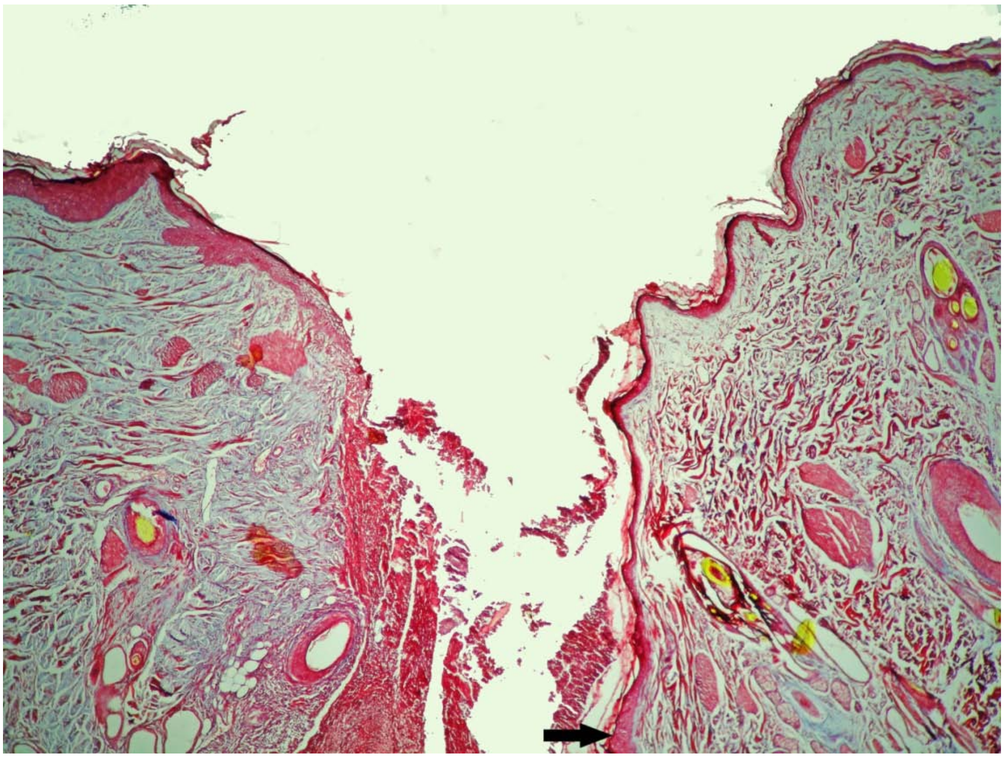


Figure 3.E. Masson's trichrome stain of wound tissue at day 7 after wounding. Representatives tissues from the treated wounds. The PRF-treated wound at day 7 has abundant newly organized collagen bundles at the wound junction. The arrow shows the healing site, 40×.



Figure 3.F. Masson's trichrome stain of wound tissue at day 7 after wounding. Representatives tissues from the untreated wounds. New collagen fiber bundles are Sparse. The arrow shows the healing site, 40×.

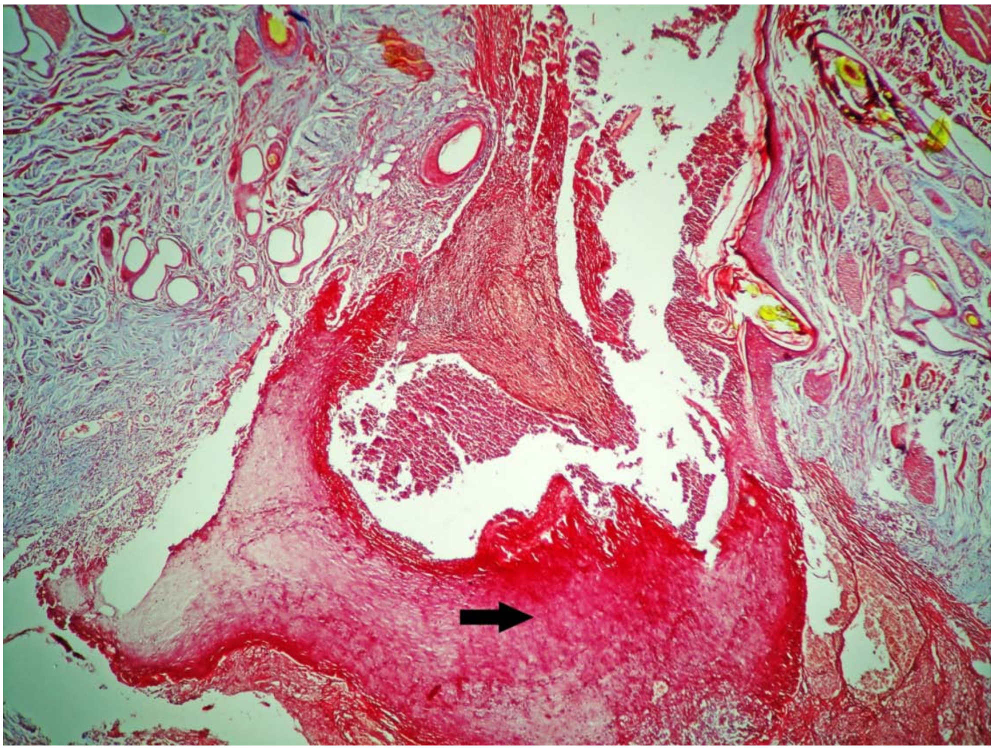


Figure 3.F. Masson's trichrome stain of wound tissue at day 7 after wounding. Representative tissues from the treated wounds. The PRF-treated wound at day 7 has abundant newly organized collagen bundles at the wound junction. The arrow shows the healing site, 40×.

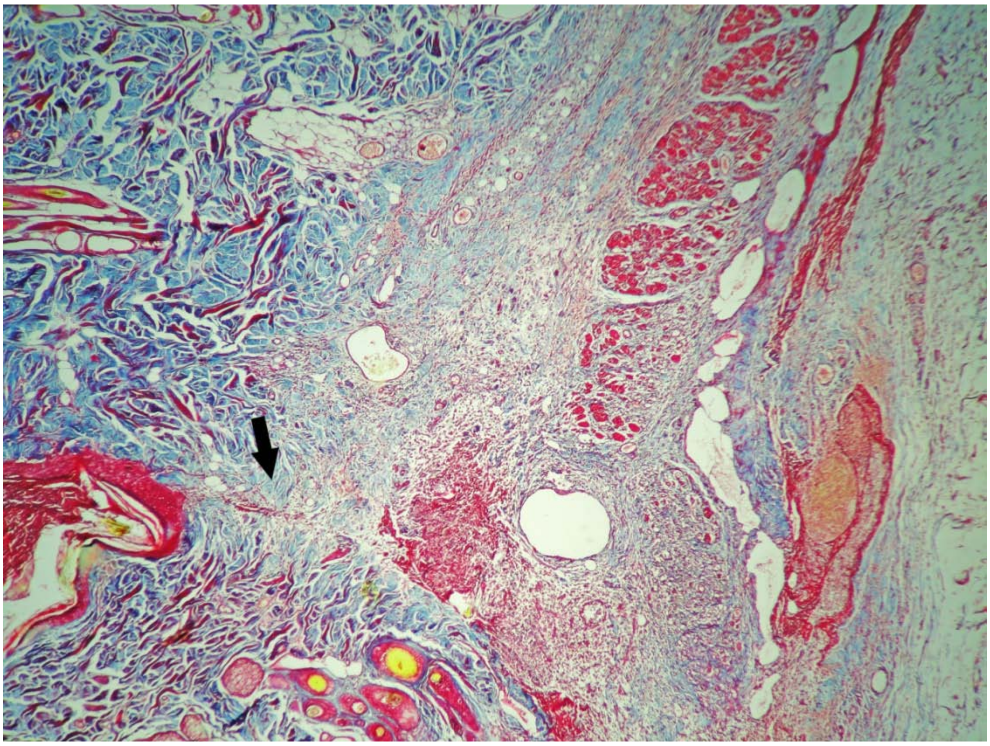


Figure 3.H. Masson's trichrome stain of wound tissue at day 7 after wounding. Representative tissues from the untreated wounds. New collagen fiber bundles are Sparse. The arrow shows the healing site, 40×.

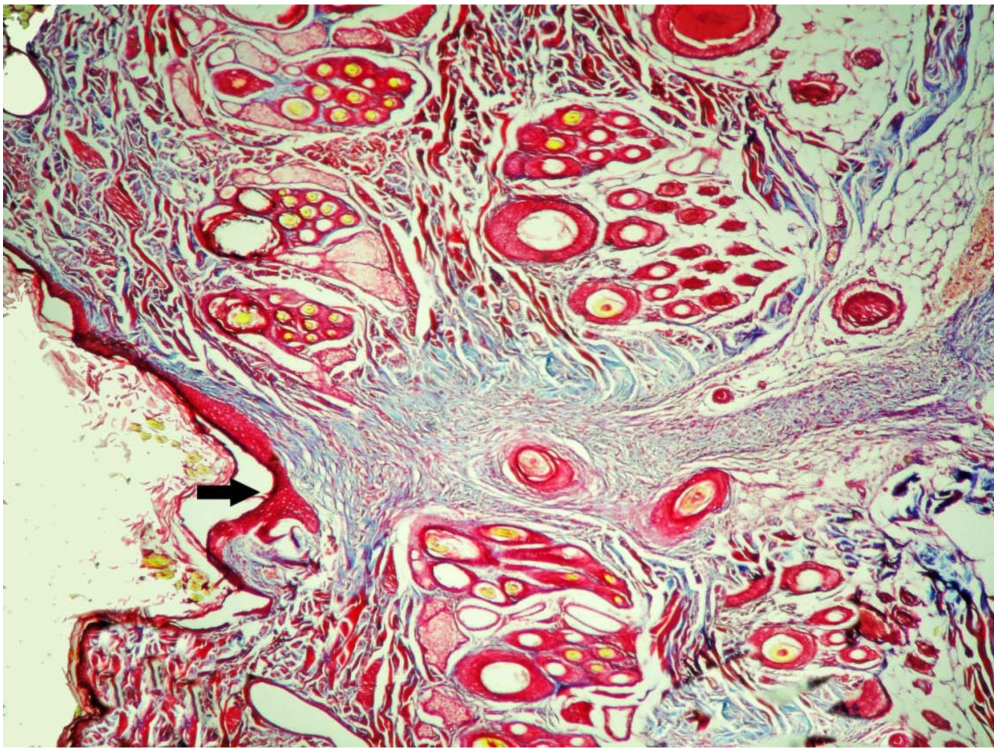


Figure 3.I. Masson's trichrome stain of wound tissue at day 14 after wounding. Specimen from the treated wounds. New collagen bundles filled densely. The arrow shows the healing site, 40×.

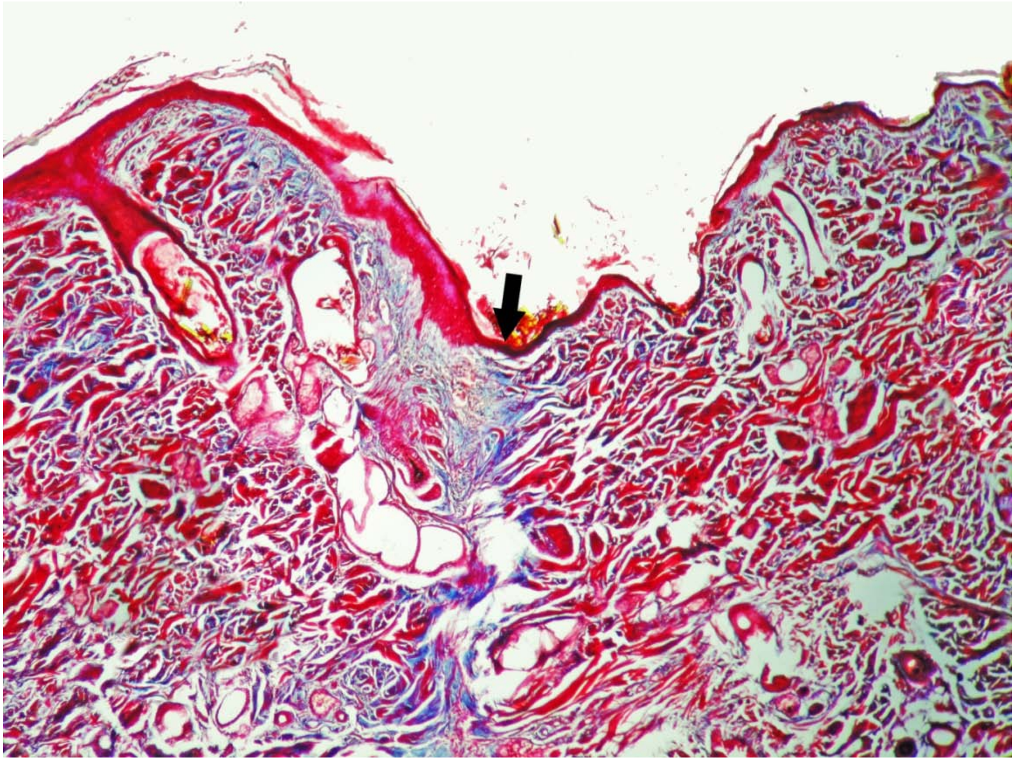


Figure 3.J. Masson' s trichrome stain of wound tissue at day 14 after wounding. Specimen from the untreated wounds. The wound junction is filled with dense collagen bundles. The arrow shows the healing site, 40×.

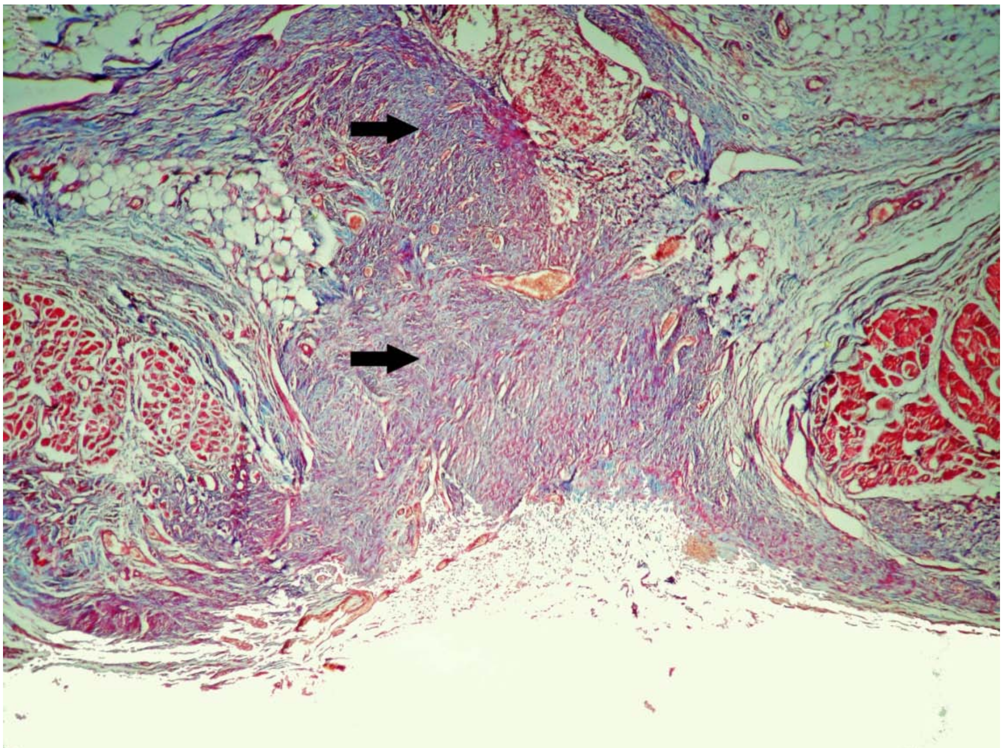


Figure 3.K. Masson' s trichrome stain of wound tissue at day 14 after wounding. Specimen from the treated wounds. New collagen bundles filled densely. The arrows show the healing site, 40×.

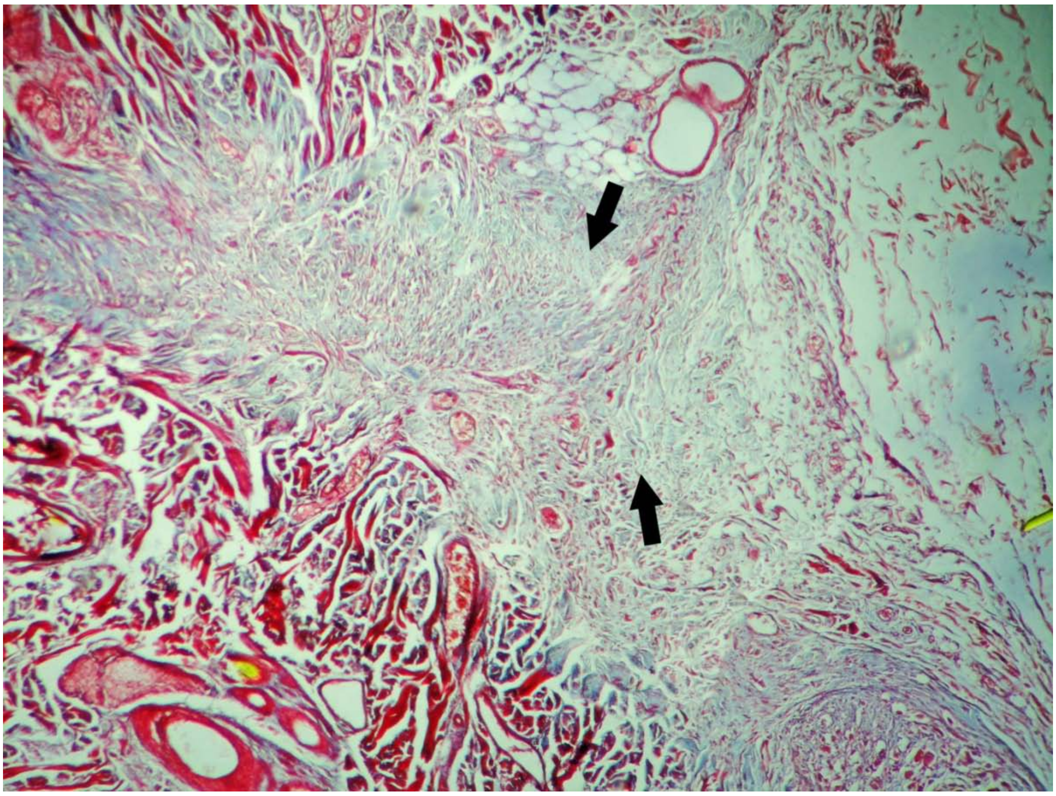


Figure 3.L. Masson's trichrome stain of wound tissue at day 14 after wounding. Specimen from the untreated wounds. The wound junction is filled with dense collagen bundles. The arrows show the healing site, 40×.

Discussion

Platelets play a key role in haemostasis and wound-healing after tissue damage. Immediately after wounding they activate fibrinogen and form a fibrin clot, thus acting haemostatic and as a tissue sealant. They also play an important role in the following stages of tissue repair. These longer lasting effects of platelet activation are caused by the expression of more than 30 growth factors. Platelets are chemotactic and induce the proliferation of fibroblasts, endothelial cells and progenitor cells, regulating the process of wound-healing (Pallua *et al.*, 2010). Platelets which are a natural source of growth factors and cytokines promote wound healing by releasing growth factors and cytokines such as platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF), platelet-derived epidermal growth factor (EGF), platelet-derived angiogenesis factor, platelet factor 4 (PF4) and platelet-activating factor (PAF) (Kimura *et al.*, 2005; Raja *et al.*, 2008; Pallua *et al.*, 2010). In addition, platelets express proteinases which trigger the release of proteolytic enzymes by other cell types playing a part in the degradation of basement membrane and extracellular matrix. As in normal platelet deposition, degranulation of the alpha granules in the platelets releases the pre-packaged growth factors. The active secretion of these cytokines is triggered by the clotting cascade of blood and starts within 10 min after aggregation. Platelet-rich fibrin (PRF) is a fibrin with a higher concentration of platelets than baseline. No definition exists as to that absolute number required, generally they are increased up to 3–5 times. PRF can be applied directly into a lesion as a matrix for regeneration. As an immediate effect, PRF will provide more rapid haemostasis and tissue adhesion by forming a fibrin clot, similar to

fibrin glue. As the amount of released factors increases with the total number of platelets delivered to a site of injury, application of PRF increases the physiologic response to a wound emulating and surpassing the ‘‘normal’’ deposition of growth factors and proteins in wound. Advocates of PRF therapy therefore claim benefits include increased tissue regeneration and a lower rate of infection, pain and blood loss (Pallua *et al.,* 2010).

Conclusion

This study demonstrates that PRF accelerates incisional wound healing. This is supported by the semi-quantitative analysis and histological demonstration of the accelerated epithelialization seen in PRF-treated incisional wounds. So we can use PRF for acceleration of epithelialization and better scar formation.

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ارزیابی هیستوپاتولوژیکی تاثیر فیبرین غنی از پلاکت بر التیام زخم های برشی پوست در سگ

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چکیده

پلاکت ها منابع طبیعی و غنی از فاکتورهای رشد و سایتوکاین های مورد نیاز جهت افزایش سرعت التیام زخم می باشند. بنابراین فیبرین غنی از پلاکت، حاوی مقادیر تغلیظ شده ای از این فاکتورها می باشد. در این مطالعه ارزیابی هیستوپاتولوژیکی و نیمه کمی تاثیر فیبرین غنی از پلاکت بر زخم های برشی پوست در سگ صورت گرفته است. حیوانات مورد مطالعه در این تحقیق عبارت بودند از پانزده سگ که در دو طرف ستون مهره های هرکدام از سگ ها یک زخم برشی به طول ده سانتی متر توسط تیغ جراحی ایجاد گردید. سپس دو لبه زخمی که در سمت چپ ستون مهره ها ایجاد شده بود پس از قرار دادن فیبرین غنی از پلاکت و دو لبه زخم سمت راست ستون مهره ها بدون قرار دادن فیبرین غنی از پلاکت بخیه زده شدند. سگ ها به سه گروه پنج تایی تقسیم شدند که نمونه های پوستی در گروه اول پس از سه روز ، گروه دوم پس از هفت روز و گروه سوم پس از چهارده روز به وسیله الکترواسکالپل برداشته شدند و سپس مورد ارزیابی هیستوپاتولوژیکی (رنگ آمیزی هماتوکسیلین ائوزین و تریکروم ماسون) و ارزیابی نیمه کمی قرار گرفتند. در نهایت ارزیابی های انجام شده نشان داد که در گروه هایی که از فیبرین غنی از پلاکت استفاده شده بود ترمیم زخم کامل تر و با سرعت بیشتری انجام می گیرد و آثار باقیمانده از زخم به طور چشمگیری کاهش می یابد.

واژگان کلیدی: فیبرین غنی از پلاکت، التیام زخم، زخم پوستی